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## Metabolic engineering of *Bacillus subtilis* for terpenoids production

Xue, Dan

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## **Summary and future perspectives**



## Abstract

Terpenoids represent a large collection of naturally produced chemicals of which many play an important role to produce pharmaceuticals. The antimalarial drug artemisinin and the chemotherapeutic agent paclitaxel (Taxol<sup>®</sup>) are outspoken representatives of this class of natural products. In order to establish a stable and efficient “cell factory” for the production of terpenoids, the potential of using *Bacillus subtilis* as a safe host was researched in this thesis. The effect of systematic overexpression of the genes involved in the methylerythritol phosphate (MEP) pathway, which upregulates the building block for terpenoid compounds, isoprene, was investigated in *B. subtilis*. It was shown that the production of carotenoids, a C<sub>30</sub> terpenoid, can be increased significantly by overexpressing the MEP pathway enzymes. Then, the segregational and structural stability of the *B. subtilis* host overexpressing the MEP pathway enzymes were further evaluated. A strain overexpressing eight genes of the MEP pathway on a plasmid clearly produced the highest level of C<sub>30</sub> carotenoids. The level of transcription for each gene in the operon was analyzed by RT-qPCR analysis a very sensitive method using the polymerase chain reaction. This is the first report of merging and stably expressing this large size operon (complete MEP pathway) from a plasmid-based system in *B. subtilis*. Moreover, this study demonstrates the possibility to express the terpene cyclase, amorphaadiene synthase (ADS), in *B. subtilis* in combination with overexpression of MEP pathway enzymes leading to the production of a considerable yield of amorphaadiene, the precursor of artemisinin.

## Summary

Terpenoids, as the most diverse group of small-molecule natural products, play an important role in the pharmaceutical, food and chemical industries. For instance, paclitaxel is used to treat cancer, artemisinin is an antimalarial drug, carotenoids have antioxidant activity, whereas volatile monoterpenes are used as flavors and fragrances. Along with an extensive usage of and demand for terpenoids products, great interest has been expressed in biotechnological production of terpenoids.

There are more than 60,000 terpenoids compounds and despite this great diversity of their structures, all of them are derived from isoprene ( $C_5$ ) units. In terpenoid biosynthetic pathways, IPP and DMAPP ( $C_5$  unit, diphosphate isoprene forms) are the basic terpenoid building blocks. For decades, isoprene yield has been considered the bottleneck for all terpenoid biosynthesis. Thus, to construct a cell platform, which can produce and tolerate high amounts of isoprene and downstream intermediates is crucial.

In the past 20 years, most research on improving the biosynthesis of terpenoid has focused on *E. coli* and *S. cerevisiae*, because both of them are fully amenable to genetic modifications and have vast molecular resources. However, our literature survey in **Chapter 2** shows that terpenoids are naturally more prevalent in *Bacillales*. The methylerythritol phosphate (MEP) pathway, which could produce the building blocks of terpenoids biosynthesis, is presented in *Bacillus subtilis* natively. In addition, several terpenoids downstream biosynthesis enzymes are endogenously in *B. subtilis*, for instance, farnesyl pyrophosphate synthase (FPPS). Furthermore, *B. subtilis* is a generally recognized as safe organism (GRAS) and has long been used for the industrial production of proteins. Attempts to biosynthesize terpenoids in this bacterium have aroused much interest in the scientific community.

In this research, the potential of *B. subtilis* as cell factory for terpenoids production was evaluated, and the ability of *B. subtilis* to accumulate divers terpenoids was exploited by overexpressing MEP pathway genes and heterologous genes from interesting biosynthetic pathways. Moreover, a stable system for further fine tuning of the MEP pathway in *B. subtilis* aiming at a more efficient production of terpenoids was established and described

in this thesis. Options for upscaling the use of the new *Bacillus* host strains have been explored and are discussed in this thesis.

### **MEP pathway engineered *B. subtilis* is a promising microbial host for terpenoid biosynthesis**

Since *B. subtilis* possesses all of the eight MEP pathway enzymes and can naturally produce high amounts of isoprene, it appears to be an ideal choice to utilize overexpression of these enzymes to increase isoprene production. However, most of the MEP pathway studies up to now have been focused on *E. coli*. For *E. coli*, the complete MEP pathway has been elucidated, all genes involved have been determined, and their corresponding enzymes were described. The story is different for *B. subtilis* where only a few studies have explored the MEP pathway leaving many questions yet to be answered. Besides that, the low number of reports about using the *B. subtilis* MEP pathway to produce terpenoids highlights the need for more research in this area.

In **Chapter 3**, we examined the effect of MEP pathway modulation on the production of terpenoids in *B. subtilis*, and systematically analyzed a series of synthetic operons expressing a specific selection of the respective enzymes from the *B. subtilis* MEP pathway. The level of production of C<sub>30</sub> carotenoids was used as a quantitative readout system to assess the effect of such modulations, since it had already been shown that the endogenous MEP pathway can be used for carotenoid production in *B. subtilis* 168. From our studies, it is clear that an increase in the formation of carotenoids happens when the level of isoprenoid precursors in *B. subtilis* 168 cells is elevated by engineering the MEP pathway. Furthermore, in order to unravel the individual contribution of each of the enzymes, *B. subtilis* 168 strains were transformed with synthetic operons harboring stepwise augmented combinations of the MEP pathway enzymes encoding genes on a plasmid. Each consecutive expression of an additional enzyme involved in the MEP pathway resulted in, to varying extents, a higher amount of carotenoids detected. This implies that almost each of the enzymes has a control on the flux through the MEP pathway.

As we proved that the whole set of MEP pathway genes could be overexpressed in *B. subtilis* and used to enhance the synthesis of building blocks we decided to explore a stable expression system in *B. subtilis*. This stable system combined with specific

terpenoid synthases could in the future increase production of terpenoids. The aim of **Chapter 4** is to construct stable *B. subtilis* strains that produce valuable terpenoids compounds by overexpressing the innate MEP pathway. A plasmid-based expression strategy was explored in which two vectors, one with rolling circle replication and another with theta-replication, were examined. Different subsets of MEP pathway genes were cloned into the vectors and the genetic stability, level of gene expression and yield of terpenoids (C<sub>30</sub> carotenoids) by the corresponding strains were evaluated. A *B. subtilis* strain overexpressing the whole MEP pathway (p04SDFHCEGA) was constructed in a stable manner, and this substantially increased the production of C<sub>30</sub> carotenoids. The cloning and expression strategy described could be widely applicable for creating metabolic pathways in *B. subtilis* and form the basis of a cell factory for high value terpenoid compounds such as paclitaxel and artemisinin.

The results presented in **Chapter 5** provide evidence that the production of more types of terpenoids can be achieved when combining the MEP pathway engineered *B. subtilis* strain with a downstream terpenoid backbone biosynthetic enzyme. In this research, the amorphadiene synthase gene *ads*, which cyclizes farnesyl pyrophosphate to amorphadiene, the essential precursor of artemisinin, was inserted into the chromosome of MEP pathway engineered *B. subtilis* 168 strain and in combination with the optimization of culture medium based on a statistic design experiment and analysis, the yield of amorphadiene was dramatically enhanced.

The observed augmentation of terpenoids production, both C<sub>30</sub> carotenoids and amorphadiene, in the engineered *B. subtilis* 168 indicates that MEP pathway modulation shows a great promise to produce terpenoids. The engineered *B. subtilis* 168 strains provided by this research in combination with other terpene synthases represent an opportunity for a GRAS cell factory for the production of numerous isoprenoids.

## Future perspectives

**Modulation expression balance of MEP pathway genes to improve yield of target terpenoids**

As the kinetics parameters of the MEP pathway enzymes are still unknown, it is unclear which step represents the largest barrier to the flux through the pathway. Therefore, the lack of knowledge about the kinetic parameters of the key enzymes is the main obstacle facing metabolic engineering of the MEP pathway in *B. subtilis* to produce terpenoids. Based on the results of Chapter 3, enzymes of MEP pathway have various contribution to the flux of final target terpenoids production in *B. subtilis*. Furthermore, researches recently indicated that there might be a cytotoxicity existing in *B. subtilis* with the accumulation of prenyl diphosphate, which influence the biosynthetic pathway flux to essential terpenoids. Thus, when engineered *B. subtilis* overexpressing an endogenous MEP pathway are constructed to provide precursors for various downstream terpenoids, modulating the activities and expressions of enzymes in the pathway becomes a critical issue for minimizing the potential cytotoxicity of intermediate metabolites and improving the final yield of terpenoids. In other words, there is a significant reason for detailed investigations of the precise mechanisms or the effects of co-regulation of the enzymes in MEP pathway in *B. subtilis*. Moreover, to further increase yields of terpenoids, more optimization could be carried out by regulating expression of MEP pathway genes systematically based on experimental design and statistic modeling analysis.

### **Investigate potential of MVA pathway in *B. subtilis* to produce terpenoids**

Since MEP pathway is endogenous in *B. subtilis*, and using endogenous genes requires less genetic adaptations and has a higher chance of producing correctly folded proteins, we chose to explore the engineering of endogenous MEP pathway in *B. subtilis* rather than the heterologous MVA pathway. Recently, the strategy to import a heterologous pathway, such as the MVA pathway, to supplement the native has been successfully used for biosynthesis of various terpenoids in *E. coli* or *S. cerevisiae*, however, this approach has not been used in *B. subtilis*. Thus, in order to satisfy the increasing demand of terpenoids, it is of great interest to also engineer the MVA pathway into *B. subtilis* and to compare that to the engineered MEP pathway.

### **Optimization of production of terpenoids *in silico* and mathematical modeling**

As systems biology is the rapidly growing, building and validating *in silico* models of diverse cellular processes could be applied to quantitative predict cellular behaviors, for instance, nutrient uptake rates, maximum theoretical molar yield and thermodynamic properties. A genome-scale metabolic network reconstruction could be used for *in silico* model analysis, and these models could provide valuable and reasonably hypothetical interactions for experimental design, as well as save lots of effort for testing.

In the past decade, more and more statistical analysis and modeling techniques have become part of biosynthesis studies. For instance, in chapter 5, we successfully used response surface methodology (RSM) to optimize the fermentation processes to increase the yield of amorphaadiene. However, when it comes to some complex situation, more mathematical and computing techniques, such as, artificial intelligence, need to be developed and applied to modeling biosynthetic processing. Among those latest models, artificial neural networks (ANN) and genetic algorithm (GA) were intensively used to optimize complex biosynthesis processes due to their ability to model highly non-linear function, and even could work precisely when there is limited knowledge of system dynamic. In order to further study the mechanism and reach economical cost of terpenoids production, more powerful and advanced analysis tools should be employed.

In conclusion, this thesis systematically investigated the influence of MEP pathway genes on the flux through the isoprenoid pathway in *B. subtilis*. Furthermore, a reliable system to produce terpenoids, for example, carotenoids and amorphaadiene, in *B. subtilis*, was developed and various factors, which might affect the growth of cells and the production of target terpenoids compound, were evaluated. MEP pathway engineered *B. subtilis* is a promising cell factory for the biosynthesis of terpenoid. However, there is still lack of sufficient knowledge on the kinetic parameters of enzymes involved, on the precise mechanism of regulation of the pathway, on the synergy of enzymes and on potential cytotoxicity due to intermediate prenyl diphosphate accumulation. Thus, as the interest is continually growth in utilizing engineered microbial platforms to synthesize pharmaceutically and industrially valuable terpenoids, intensive research and further optimization is certainly necessary.



